# Epac1 and Epac2 are differentially involved in inflammatory and remodeling processes induced by cigarette smoke

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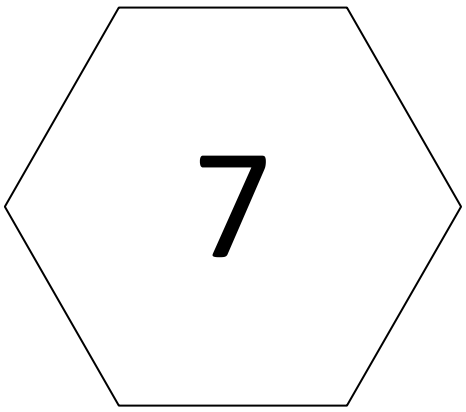


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**Abstract**

Cigarette smoke (CS) induces inflammatory responses characterized by increase of immune cells and cytokine release. Remodeling processes, such as mucus hypersecretion and extracellular matrix protein production, are also directly or indirectly induced by CS. Recently, we showed that activation of the exchange protein directly activated by cAMP (Epac) attenuates CS extract-induced interleukin (IL)-8 release from cultured airway smooth muscle cells. Using an acute model of CS exposure, we now studied the role of Epac1, Epac2 and the Epac effector phospholipase-Cε (PLCε) in airway inflammation and remodeling *in vivo.* Compared to wild-type (WT) mice exposed to CS, the number of total inflammatory cells, macrophages, neutrophils and IL-6 release was lower in Epac2-/- mice, which was also the case for neutrophils and IL-6 in PLCε-/- mice. Taken together, Epac2 acting partly via PLCε, but not Epac1, enhances CS-induced airway inflammation *in vivo*. In total lung homogenates of Epac1-/- mice, MUC5AC and matrix remodeling parameters, such as transforming growth factor-β1, collagen I and fibronectin, were increased at baseline. Our findings suggest that Epac1 is capable to primarily inhibit remodeling processes, whereas Epac2 primarily increases inflammatory processes *in vivo*.

**Key words (3-5):** Novel cAMP effectors act pro-inflammatory and anti-fibrotic *in vivo*

## Introduction

Cigarette smoke (CS) is the main risk factor of chronic obstructive pulmonary disease (COPD) and contributes to neutrophilic inflammation and remodeling (1; 2). Next to glucocorticoids and anticholinergics, $\beta_2$-agonists and inhibitors of phosphodiesterase (PDE) 4 are currently used to alleviate COPD symptoms (3). Both $\beta_2$-agonists and PDE4 inhibitors enhance the cellular level of cyclic AMP (cAMP) either by $G_s$-protein-coupled receptor-mediated activation of cAMP-producing adenylyl cyclase, or by inhibition of cAMP-degrading PDEs, respectively. Subsequently, cAMP activates two main effectors, protein kinase A (PKA) and the exchange protein directly activated by cAMP (Epac). The two isoforms of Epac, Epac1 and Epac2, have their own subset of effectors and expression pattern resulting in diverse biological functions depending on the cell type involved (4).

We reported in cultured human airway smooth muscle cells that specific pharmacological activation of either PKA or Epac reduces the inflammatory response induced by CS (5). In rat alveolar macrophages, activation of Epac, but not PKA, inhibited the FcR-mediated phagocytic activity (6). Furthermore, adenovirus-mediated gene transfer of Epac1 inhibited TGF-β1-induced collagen synthesis in cardiac fibroblasts (7). In turn, TGF-β1 reduced Epac1 expression (7), indicating that the loss of Epac1 may contribute to cardiac fibroblast remodeling. Inflammation and remodeling may further be mediated by the Rap-activated phospholipase Cε (PLCε), a direct effector of Epac (8; 9). PLCε is highly expressed in the mouse lung (10), but its function herein is undefined yet. PLCε positively regulated proliferation of dermal fibroblasts (9), a major source of matrix protein production. In the skin, PLCε stimulated pro-inflammatory mediator production including keratinocyte-derived chemokine (KC), the murine functional homolog of interleukin (IL)-8 (11), IL-1β and tumor necrosis factor (TNF)-$\alpha$ (12). The PLCε-mediated increase in KC was accompanied by neutrophilia (11). Taken together, Epac1, Epac2 and PLCε seem to regulate remodeling and inflammation, which may depend on the Epac isoform or downstream effectors.

The specific role of Epac1, Epac2 and the Epac effector PLCε, however, in inflammation and remodeling of the airways in an acute model of CS exposure is

unknown. In this study, we analyzed the effect of 4 day exposure to CS on inflammatory and remodeling parameters in lungs of wild-type, Epac1-/-, Epac2-/- and PLCε-/- mice.

## Materials & Methods

### Animals and animal model

C57Bl/6J wild type animals and Epac1-/-, Epac2-/- (13) and PLCε-/- mice were used for all experiments. Female mice, n= 6-10 per group (female, 9-21 weeks of age) were exposed to the smoke of filter-free Kentucky 3R4F research cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, USA) by whole body exposure in a 6L Perspex box as previously described (14). On day 1, mice were exposed to the smoke of one cigarette in the morning and three cigarettes in the afternoon. On day 2, 3 and 4, mice were exposed to the smoke of five cigarettes in the morning and five cigarettes in the afternoon. Control animals were exposed to air. Mice were sacrificed on day 5 by intraperitoneal pentobarbital injection (400 mg/kg). Lungs were lavaged and lung tissue was collected for RT-PCR and Western blot analysis. All experiments were approved by the University of Groningen Committee for Animal Experimentation.

### Bronchoalveolar lavage fluid (BALF)

Lungs were washed via a tracheal cannula with 1 ml PBS containing 5% bovine serum albumin (BSA) and a mix of protease inhibitors (F. Hoffman-La Roche, Basel, Switzerland) followed by four steps with 1 ml PBS only. Cells from all fractions were collected by centrifugation (200 g, 10 minutes, 4°C). Supernatants of the first wash step were collected for analysis of cytokine levels (KC, IL-6, IL-17, TNF-α, vascular endothelium-derived growth factor (VEGF), IL-1β, macrophage inflammatory protein-1α (MIP-1α)) using a MILLIPLEX assay (Millipore, Billerica, USA) according to manufacturer's protocol. Cells from all 5 fractions were combined and resuspended in 200 µl PBS. After determining total cells numbers, $5*10^5$ cells were spun on glass coated with PBS containing 3% BSA. Cytospins were stained with May-Grünwald Giemsa (Sigma, St Louise) and macrophages, neutrophil and lymphocyte numbers were determined by counting 400 cells in duplicate (14).

### Genotyping

DNA was isolated from mouse ear using NucleoSpin Tissue kit (Machery Nagel, Düren, Germany) according to manufacturer's instructions. Using the primers

listed in Table 1, DNA was amplified using HotStar Taq Master Mix (Qiagen, Valencia, CA, USA). To verify complete knockdown of Epac2 and PLCε, two PCR reactions were performed to identify the wildtype DNA of the gene and the knockout DNA, respectively. One PCR reaction with three primers was used to confirm Epac1 knockdown. After the PCR reaction, the samples were loaded on a 1 or 2% agarose gel to identify DNA products.

**Table 1:** Primers used for genotyping

| Primers | PCR reaction | | Sequence 5'- 3' |
|---|---|---|---|
| Epac1 | Wild-type DNA | Flox5' forward | GTTTGCCTGCCTGAATGTCT |
| | Knockout DNA | Flp reverse | AAGGAGGAAGCAGGAGCAAGATACAGG |
| | | 3'endo/exon8 reverse | CATGAAGCAAAGACAGTTGACATC |
| Epac2 | Wild-type DNA | Forward | TGAACAGATTTGTGACCGGAT |
| | | Reverse | CTGATCACATTAGCAAGCTC |
| | Knockout DNA | Forward | GCATACATTATACGAAGTTATC |
| | | Reverse | CTGATCACATTAGCAAGCTC |
| PLCε | Wild-type DNA | Forward | GCGTATTTCCAGAGTTAGAACAAGG |
| | | Reverse | CCACAACCAGGACCAGAGATG |
| | Knockout DNA | Forward | GCGTATTTCCAGAGTTAGAACAAGG |
| | | Reverse | CTGCAAAGGGTCGCTACAGA |

**Real time quantitative RT-PCR**

RNA from each mouse was collected from frozen lung using Nucleospin RNA II kit (Machery Nagel, Düren, Germany) according to the manufacturer's instructions. cDNA was prepared from equal amounts of RNA followed by a real-time qPCR (Westburg, Leusden, The Netherlands) using the forward and reverse primers listed in Table 2. Expression of all target genes mRNA was normalized against the expression of 18S.

**Table 2:** Primers used for RT-PCR

| Primers | | Sequence 5'- 3' |
|---|---|---|
| 18S | Forward | AAACGGCTACCACATCCAAG |
| | Reverse | CCTCCAATGGATCCTCGTTA |
| AC2 | Forward | GGAGATCGAAACCATGGAGA |
| | Reverse | CTGAACTTCGGCTTGGAAAG |
| AC9 | Forward | CTTCAGCTCCCTTCTGGATG |
| | Reverse | GATCCATTCCAGCAACCACT |
| $\beta_2$-AR | Forward | GGTTATCGTCCTGGCCATCGTGTTTG |
| | Reverse | TGGTTCGTGAAGAAGTCACAGCAAGTCTC |
| Collagen I | Forward | CACCCTCAAGAGCCTGAGTC |
| | Reverse | GTTCGGGCTGATGTACCAGT |
| Epac1 | Forward | GCGTAATACGACTCACTATAGGGAGAGAGCTGCAGT ACTGGGTG |
| | Reverse | GCGTAATACGACTCACTATAGGGAGACAGCTGCTGG ACATAAGC |
| Epac2 | Forward | GCGTAATACGACTCACTATAGGGAGAGACTGTGGAT GACCTAGAG |
| | Reverse | GCGTAATACGACTCACTATAGGGAGACAAGGCGTATT GTTTCTAG |
| Fibronectin | Forward | ACCACCCAGAACTACGATGC |
| | Reverse | GGAACGTGTCGTTCACATTG |
| IL-13 | Forward | CAGCATGGTATGGAGTGTGG |
| | Reverse | AGGCCATGCAATATCCTCTG |
| MUC5AC | Forward | GAGATGGAGGATCTGGGTCA |
| | Reverse | GCAGAAGCAGGGAGTGGTAG |
| PDE3B | Forward | CCAATTCCTGGCTTACCTCA |
| | Reverse | GTGATCGTAATCGTGCATGG |
| PDE4D | Forward | GGAGGACAATCGTGAGTGGT |
| | Reverse | CAAGTTTCAGGCTGGCTTTC |
| PKA-RIa | Forward | TTTGGAGAGCTGGCTTTGAT |
| | Reverse | TGCATCGGCTACTGTGAGAC |

| Primers | | Sequence 5'- 3' |
|---|---|---|
| PKA-RIIa | Forward | AGTGACTCGGACTCGGAAGA |
| | Reverse | TTCAAACATGGCATCCAGAA |
| PKA-C | Forward | CAACCGCATTTATGGCTTCT |
| | Reverse | AGGAGACACCACGGTCATTC |
| PLCε | Forward | CAGAGCCCTTTGCTGTTTTC |
| | Reverse | TTCTGGACCCACAGCTCTCT |
| TGF-β1 | Forward | TGAGTGGCTGTCTTTTGACG |
| | Reverse | TCTCTGTGGAGCTGAAGCAA |

AC, adenylyl cyclase; $\beta_2$-AR, $\beta_2$-adrenoceptor, Epac, exchange protein directly activated by cAMP; PDE, phosphodieasterase; PKA-C, PKA catalytic subunit; PKA-RIa and RIIa, PKA regulatory subunit RIa and RIIa; TGF-β1 transforming growth factor β1.

**Western analysis**

Frozen lung tissue was pulverized in liquid nitrogen and homogenized in sodium dodecyl sulfate (SDS) lysis buffer (62.5 mM Tris, 2% wt/vol SDS, 1 mM NaF, 1 mM $Na_3VO_4$, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 7 µg/ml pepstatin A, pH 6.8). After centrifugation, equal protein amounts in the supernatants were loaded on a SDS-Page gel, followed by transfer to a nitrocellulose membrane. After blocking with 5% milk powder in Tris buffered saline with 0.1 % Tween 20 for 2 h, membranes were incubated with the appropriate antibody at 4°C overnight, followed by 2 h incubation with the appropriate secondary antibody. Antibodies and dilutions are listed in Table 3. Bands were visualized by addition of western lighting plus-ECL (Perkin Elmer Inc., Waltham, MA, USA).

**Table 3:** Antibodies used for Western blot and immunohistochemistry

| Target | Source | Dilution | Secondary antibody |
|---|---|---|---|
| αSMA | Abcam | 1:200 | Rabbit (1:200) |
| β-actin | Santa Cruz | 1:1000 | Mouse (1:2000) |
| Collagen 1 | Southern Biotech | 1:1000 | Goat (1:10,000) |
| Epac1 | Cell Signaling | 1:500 | Mouse (1:2000) |
| Epac2 | Cell Signaling | 1:500 | Mouse (1:2000) |
| Fibronectin | Santa Cruz | 1:250 | Goat (1:10,000) |
| GAPDH | Santa Cruz | 1:2000 | Mouse (1:2000) |
| SPDEF | Biorbyt | 1:100 | Rabbit (1:200) |

Secondary antibodies were obtained from Sigma Aldrich, Buchs SG**,** Switzerland**.** Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cell Signaling, Danvers, MA, USA; Southern Biotech, Birmingham, Alabama, USA; Biorbyt, Cambridge, UK, Abcam, Cambridge, UK.

**MUC5AC ELISA**

MUC5AC protein expression in BALF was determined using ELISA. A 96 wells maxisorb plate (ThermoScientific, Roskilde, Denmark) was coated overnight with MUC5AC antibody (NeoMarkers, Fremont, CA, USA; 0.05 µg/well), followed by blocking for 2 h with 2% BSA. 100 µl BALF was added per well. After 75 minutes, soybean agglutinin-HRP (Sigma Aldrich, Buchs SG**,** Switzerland) was added as a secondary antibody followed by a substrate reaction with 3,3',5,5'-tetramethylbenzidine (Merck, Whitehouse Station, NJ, USA; 0.1µg/ml) and absorbance was determined at 450 nm.

**Immunohistochemistry**

Lung tissue was dissected and fixed with 4% paraformaldehyde in PBS, dehydrated, and embedded in paraffin according to standard methods (15). Goblet cells were identified using Periodic acid-Schiff (PAS) staining. Lung sections were deparaffinised and hydrated followed by 15 minutes incubation in 1% periodic acid solution (Sigma Aldrich, Buchs SG**,** Switzerland). After a wash step, Schiff's reagent (Sigma Aldrich, Buchs SG**,** Switzerland) was added for 30 minutes

in the dark. Nuclei were stained using Mayer's hematoxylin solution (Sigma Aldrich, Buchs SG**,** Switzerland) for 10 minutes. Lung sections were rehydrated and goblet cells were analyzed. Immunostaining for SPDEF (SAM pointed domain ETS factor) was performed using a polyclonal rabbit anti-SPDEF antibody (Biorbyt, Cambridge, UK). Slides were deparaffinized and antigen retrieval was performed in 10mM Tris/1mM EDTA buffer (pH= 9.0) under 125° for 15 minutes. Slides were washed and then incubated with 0.3% $H_2O_2$ for 30 minutes. After wash steps, slides were incubated with SPDEF antibody at 1:100 for 1h. Second *horseradish peroxidase* (HRP) conjugated polyclonal goat-anti-rabbit antibody was used at 1: 200 for 30 minutes. Then 3,3'-Diaminobenzidine (DAB) was used for color reaction and hematoxylin solution was for nuclei staining. Semiquantification of the staining intensity was performed in triplicate in three classes:1) low,2) medium, and 3) high levels of SPDEF, essentially as reported previously (16). A comparable method was used for immunostaining of α-smooth muscle actin (αSMA). Slides were blocked with 0.3% $H_2O_2$ for 30 minutes, followed by 1 h incubation with αSMA (1:200) (Abcam, Cambridge, UK). Goat-anti-rabbit antibody (1:200) (Dako, Denmark) was added for 30 minutes. For the colour reaction DAB was used and hematoxylin solution was used for nuclei staining. To identify collagen slides were deparaffinized and incubated in sirius red solution for 1 h followed by a 2 minutes wash in 0.01 M HCl. Slides were counter stained with hematoxylin solution.

**Epithelial wall thickness**

To analyze epithelial wall thickness total airway area and lumen area was measured. Epithelial wall thickness was calculated as previous described (17).

**Statistical analysis**

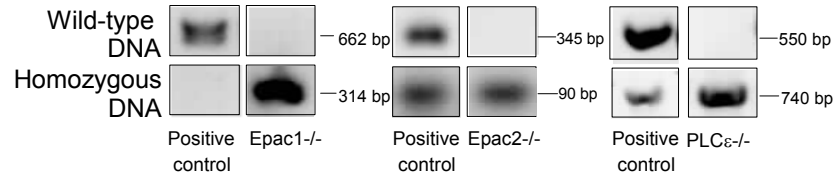
Data are expressed as mean ± standard error of the mean (SEM). Two-way ANOVA, followed by a Newman-Keuls comparison test or a Mann Whitney Rank Sum test was used as appropriate to identify statistical differences ($p<0.05$).

## Results

### Basal characterization of WT and knock out mouse

The characterization of the genotypes of the Epac1-/-, Epac2-/- and the Epac effector PLCε-/- mice was performed on DNA level (Supplemental Fig. 1A) and confirmed knock down of the appropriate gene in all mouse strains. Epac1 protein was absent in Epac1 -/- mice ($p<0.001$), whereas Epac2 protein was not altered (Supplemental Fig. 1B). Expression of Epac2 protein, but not Epac1 protein, was largely blunted in Epac2-/- mice ($p<0.001$; Supplemental Fig. 1B). Basal characteristics color, size and background were comparable between the four mouse strains (Fig. Supplemental 2A). Staining of αSMA and total collagen in lung sections revealed no significant differences between the WT and the knockout mice at baseline, whereas αSMA tended to be reduced in Epac1-/- (αSMA, $p=0.12$). The epithelial wall thickness was significantly lower in the Epac1-/- and PLCε-/- compared to WT mice, whereas expression of the transcription factor for mucus secretion SPDEF tended to be increased in Epac1-/- ($p=0.065$) (Supplemental Fig. 2B&D).

A

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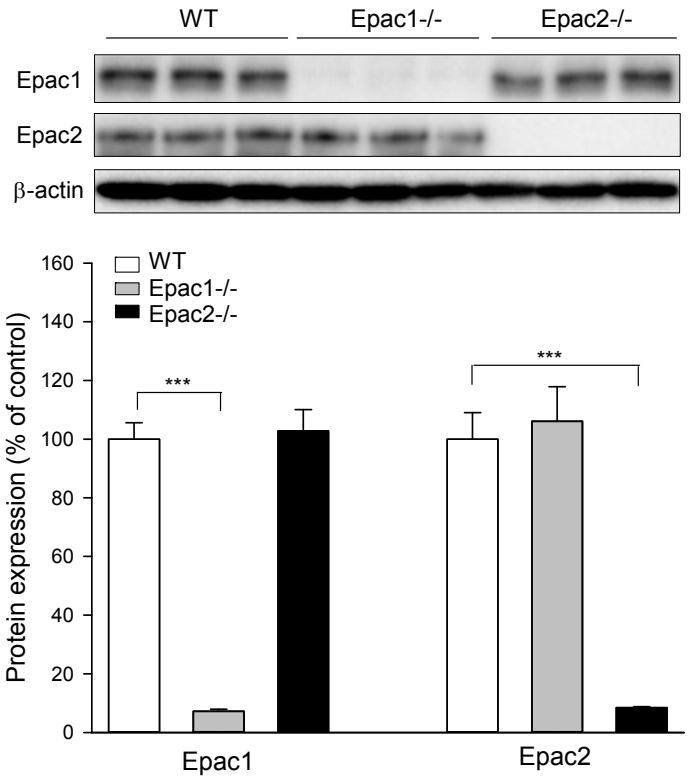
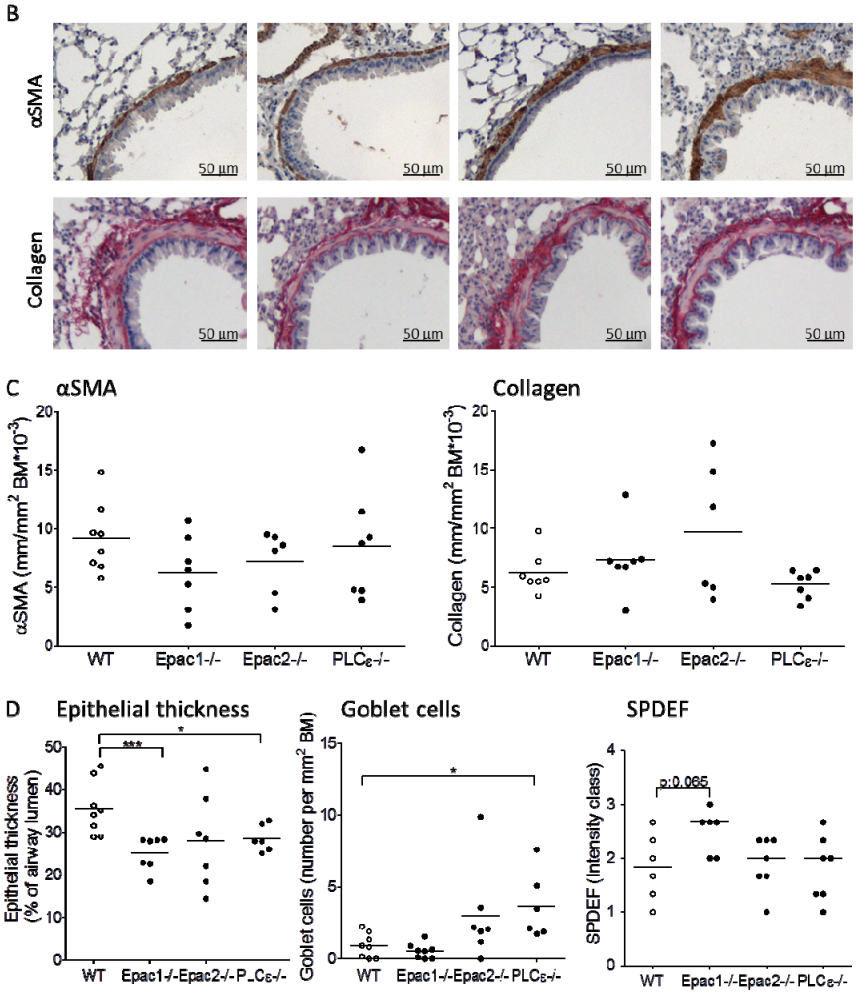



**Supplemental Figure 1:** Characterization of genotypes of Epac1-/-, Epac2-/- and PLCε-/- mice. DNA analysis (A) on mouse ear on Epac1-/-, Epac2-/- and PLCε-/- mice. Epac1 and Epac2 protein analysis on lung tissue (B) was performed in WT, Epac1-/- and Epac2-/- mice.

A

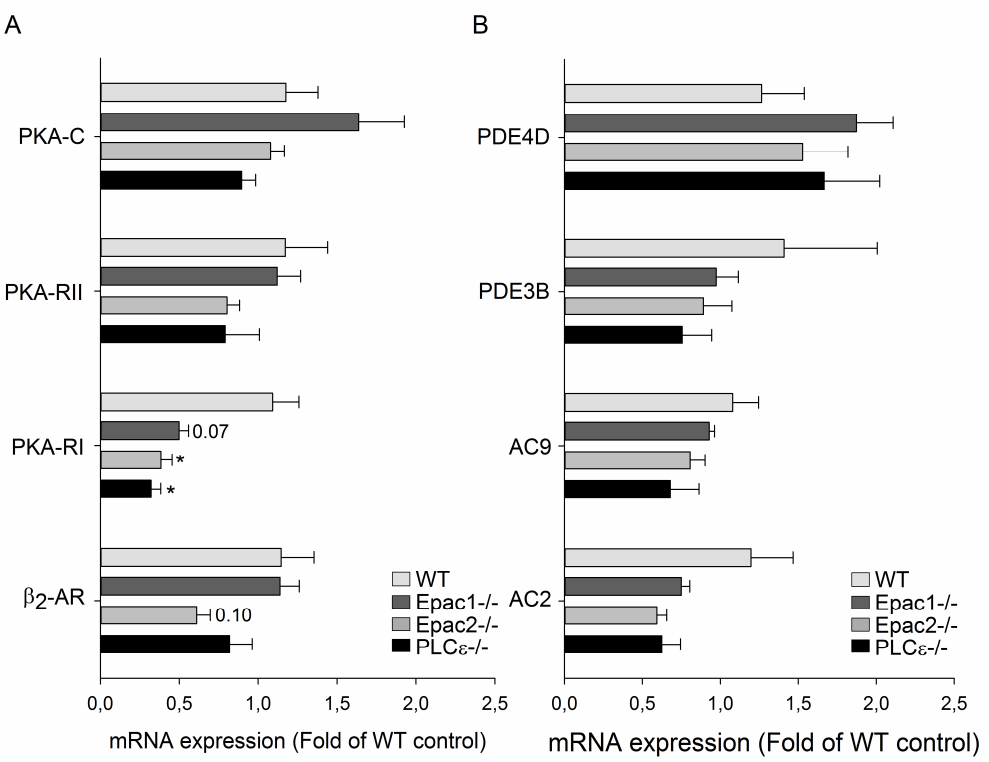
| Characteristic | WT | Epac1-/- | Epac2-/- | PLCε-/- |
|---|---|---|---|---|
| Background | C57Bl/6J | C57Bl/6J | C57Bl/6J | C57Bl/6J |
| Colour | Black | Black/agouti | Black | Black |
| Average litter size (m/f) | Ordered from Harlan | 3.25 / 3.25 | 2.6 / 2 | 2.4 / 2 |
| Size | Not altered | Not altered | Not altered | Not altered |




**Supplemental Figure 2:** Basal characteristics of Epac1-/-, Epac2-/- and PLCε-/- mice (A). Representative pictures of α-smooth muscle actin (αSMA) and collagen staining (B) are shown. Quantification of αSMA and collagen in lung sections (C). In addition, epithelial thickness, goblet numbers and SPDEF staining were analyzed (D). BM: Basement membrane. $^{*}$p<0.05, $^{***}$p<0.001 compared to WT.

**The cAMP pathway**

We analyzed the basal expression of components of the cAMP pathway in the four mouse strains and identified a significant reduction of PKA-RI mRNA in Epac2-/-, PLCε-/- compared to WT mice, whereas a trend towards reduction was observed in Epac1-/- mice (Fig. Supplemental Fig. 3). The expression of other components was not altered (Fig. Supplemental Fig. 3). The effect of 4 day exposure to CS on mRNA expression of different components of the cAMP pathway was tested in WT mice. Besides Epac1 and Epac2, PKA-RI, PKA-RII, PKA-C and PLCε, $\beta_2$- adrenoceptor ($\beta_2$-AR), PDE4D, PDE3B and adenylyl cyclase (AC) subtypes 2 and 9 were chosen due to their association with COPD (18-22). Exposure of WT mice to CS did not significantly alter mRNA expression of $\beta_2$-AR, PKA-RI, PKA-RII, AC2, AC9, PDE3B, PDE4D, Epac1, and Epac2 (Fig. 1A), although there was a trend towards a reduction in mRNA expression for the $\beta_2$-AR and Epac1 ($p= 0.06$ and $p= 0.09$, respectively). Protein expression of Epac1 and Epac2 was not altered in WT mice after exposure to CS (Fig. 1B). Exposure of Epac1-/- to CS significantly reduced the mRNA expression of Epac2, AC2, AC9, PKA-RII, whereas there was a trend towards a reduction of mRNA expression of $\beta_2$-AR and PLCε (each $p= 0.06$) (Fig. S4). A trend towards reduction of PKA-C was observed in Epac2-/- mice after exposure to CS ($p=0.06$) (Fig. Supplemental 4).




**Supplemental Figure 3:** Determination of mRNA expression of components of the cAMP pathway in total lung homogenates. Lung tissue of WT, Epac1-/-, Epac2-/- and PLCε-/- mice was homogenized and mRNA expression of the indicated proteins was analyzed (A&B). Data are presented as mean ± SEM of 4 animals. * $p<0.05$ compared to WT.

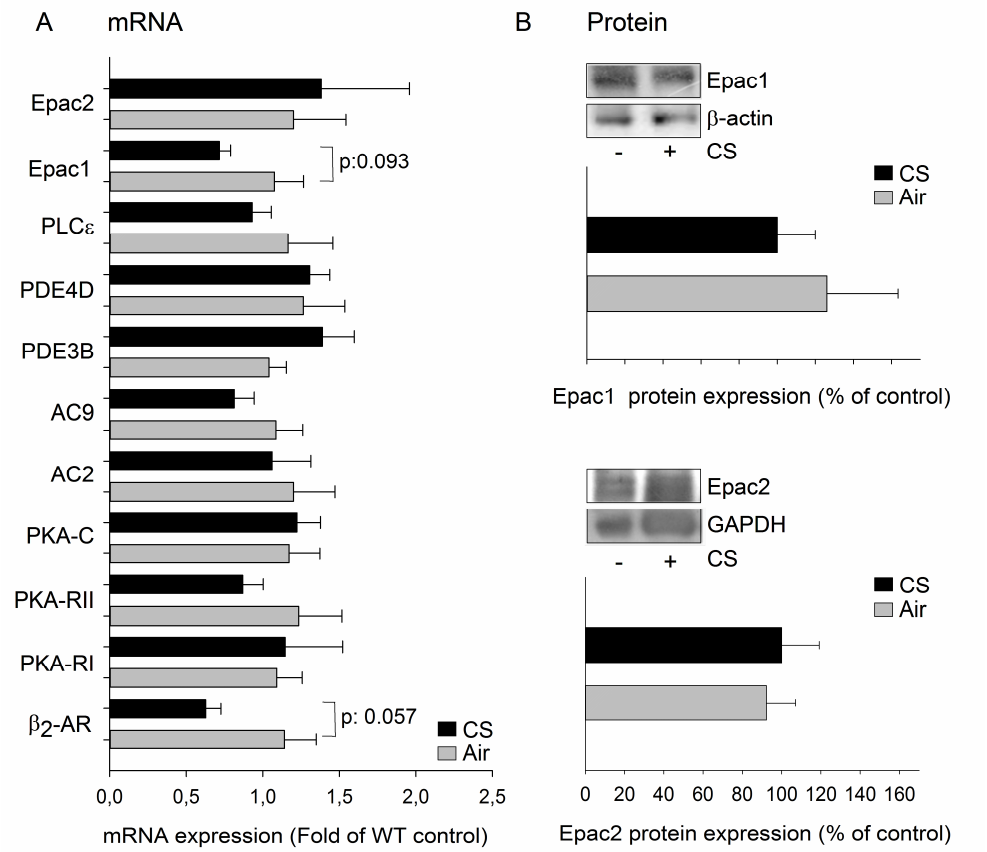
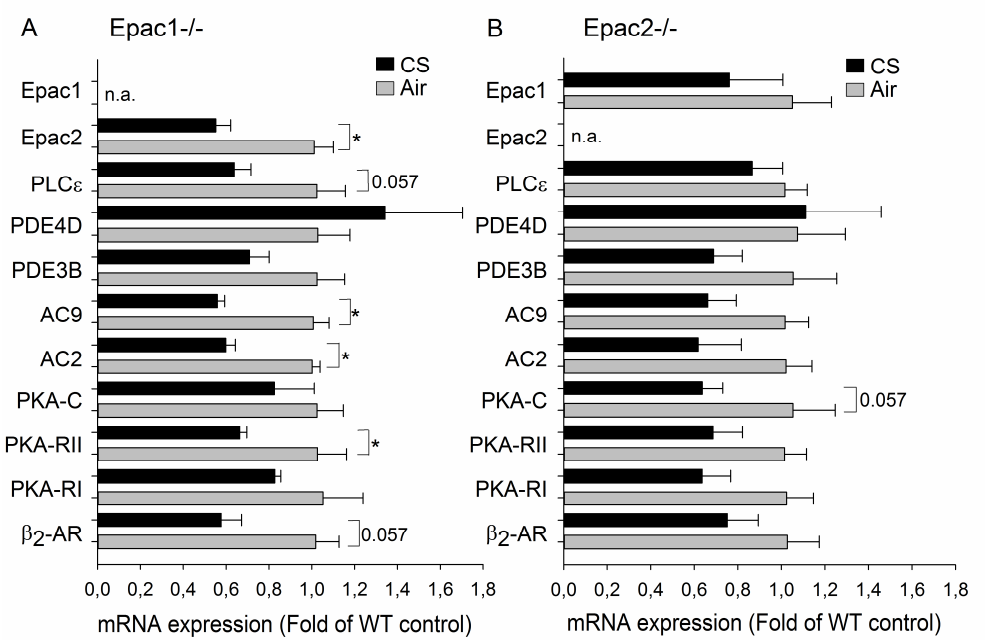



**Figure 1:** Effect of CS on different components of the cAMP pathway. Expression of the indicated proteins was measured in lung homogenates of WT mice exposed to air or CS for 4 days (A). Protein expression of Epac1 and Epac2 was measured in lung homogenates of WT mice exposed to air and CS for 4 days (B). Epac1 and Epac2 protein expression was normalized against GAPDH or β-actin. Data are presented as mean ± SEM of 6-10 animals. $^{*}p<0.05$, $^{***}p<0.001$ compared to WT.




**Supplemental Figure 4:** Effect of CS on different components of the cAMP pathway. Expression of the mRNA of the indicated proteins was measured in lung homogenates of Epac1-/- (A) or Epac2-/- (B) mice exposed to air or CS for 4 days. Data are presented as mean ± SEM of 4 animals. * $p<0.05$ compared to basal control.

**Role of Epac1-/-, Epac2-/- and PLCε-/- in airway inflammation**

In WT mice, CS exposure induced a 1.5-fold increase in the total number of inflammatory cells in BALF (Fig 2A, $p<0.01$). A similar increase was found in Epac1-/- and PLCε-/- mice (Fig 2A, $p<0.01$). In contrast, a CS-induced increase in total inflammatory cell number observed in WT mice was absent in Epac2-/- mice (Fig. 2A; $p<0.05$). No changes in total inflammatory cell numbers were observed in air exposed knockout mice compared to WT mice (Fig. 2A).

Differential cell counting revealed that more than 80% of all inflammatory cells in the CS exposed WT mice were macrophages. Exposure to CS increased macrophage numbers in PLCε-/- mice to a similar extent (Fig. 2B, $p<0.01$), an effect not being observed in Epac1-/- and Epac2-/- mice (Fig. 2B). Lymphocytes, only present in low numbers in BALF of air-exposed WT mice, were not significantly affected by CS exposure in any of the groups compared to their

appropriate air control groups (Fig. 2C). However, the basal (3.6-fold) and CS-induced (2.2-fold) numbers of lymphocytes in Epac1-/- mice were increased compared to the basal and CS-induced number in WT (Fig. 2C, $p<0.05$ both). As reported previously (14), CS exposure markedly increased neutrophils numbers by 44-fold in BALF of WT mice (Fig. 2D, $p<0.001$). A similar increase in the number of neutrophils was seen in Epac1-/- mice. In contrast, this effect was significantly reduced in Epac2-/- and PLCε-/- mice (Fig. 2D) ($p<0.05$ and $p<0.01$, respectively). No changes were observed in neutrophil numbers between any of the air exposed groups.

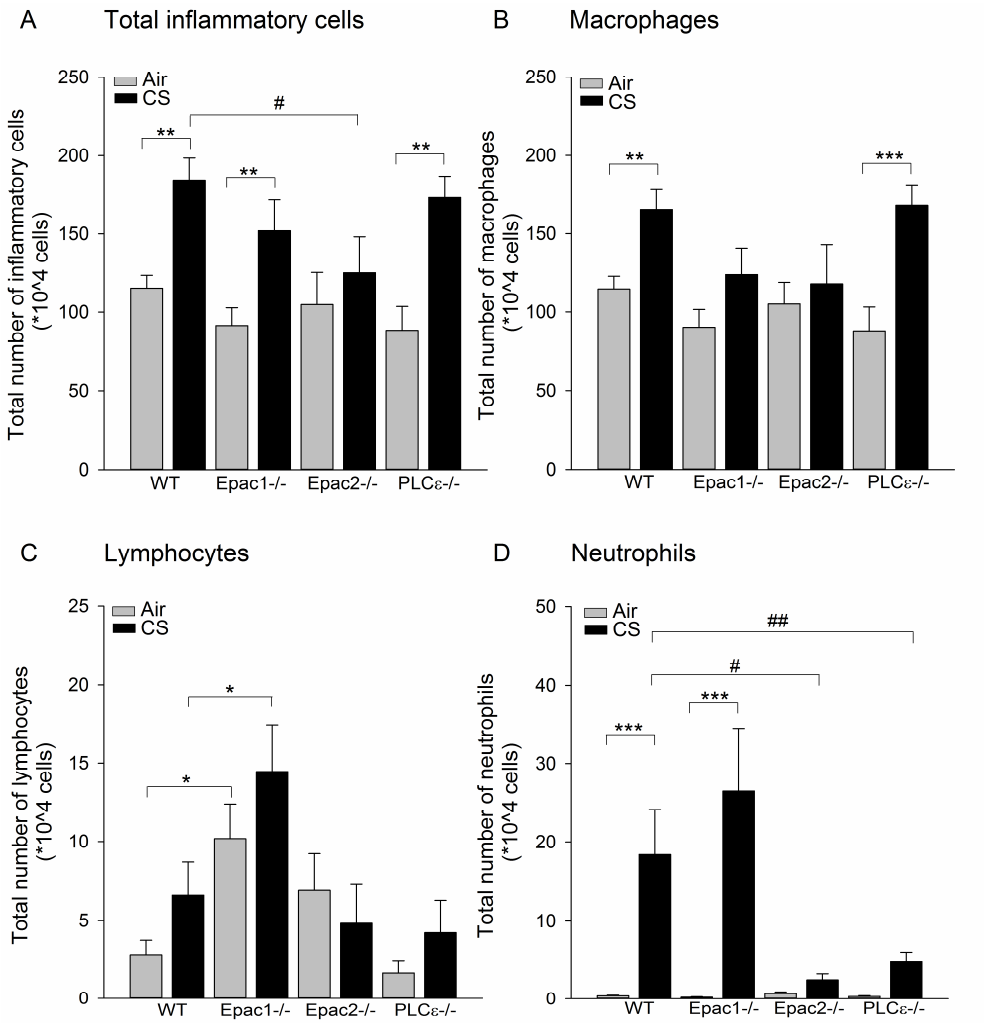



**Figure 2:** Inflammatory cell numbers were determined in BALF. WT, Epac1-/-, Epac2-/- and PLCε-/- mice were exposed to air or CS for 4 days after which BALF was collected. The level of total inflammatory cells (A), macrophages (B), lymphocytes (C) and neutrophils (D) in BALF were analyzed. Data are presented as mean ± SEM of 6-10 animals. $^{*}$ p<0.05, $^{**}$p<0.01, $^{***}$p<0.001 compared to basal control, $^{\#}$p<0.05 compared to WT exposed to CS.

The inflammatory cytokines IL-8 (in mice KC), IL-1β, IL-6, IL-13 and TNFα induce a persistent inflammatory response upon CS exposure (23-25). We determined these cytokines in BALF of WT, Epac1-/-, Epac2-/- and PLCε-/- exposed to either air or CS. As shown in Fig.3, CS induced similar KC levels in BALF of WT, Epac1-/-,

Epac2-/- and PLCε-/- mice, without any significant differences between the different strains (Fig. 3A, p<0.05 all). These KC levels significantly correlated with the number of neutrophils in WT mice (r=0.519, p=0.02) and Epac2-/- mice (r=0.729, p=0.005). No correlation was observed in Epac1-/- mice (r=-0.183, p=0.514) and PLCε-/- mice (r=0.320, p=0.227). IL-6 levels in BALF of WT and Epac1-/- mice were similarly increased after CS exposure (Fig. 3B, p<0.01), whereas no significant increase was observed in the Epac2-/- and PLCε-/- mice (Fig. 3B). The CS-induced IL-6 level was only significantly reduced in the PLCε-/- compared to WT mice (p< 0.01). The level of IL-1β was not affected by CS in the WT, Epac1-/- and PLCε-/- mice (Fig. 3C), but was significantly reduced in CS-exposed Epac2-/- mice (Fig. 3C, p<0.05). Levels of IL-13 and TNFα were not detectable (not shown). In addition, no changes in basal cytokine levels in BALF were observed between any of the air exposed groups.

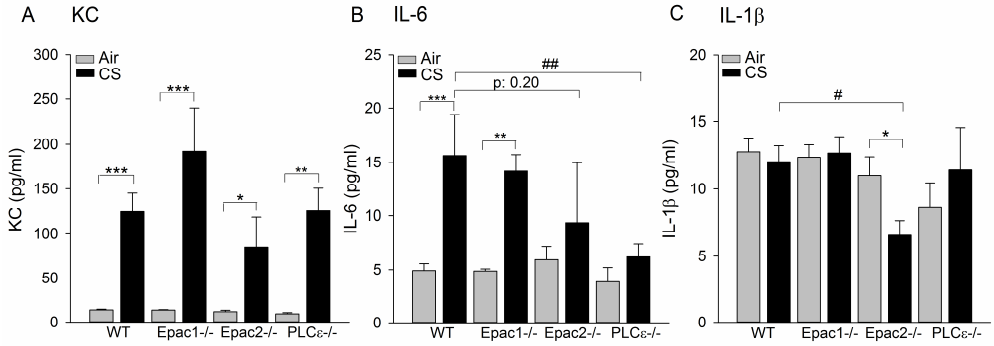



**Figure 3:** Cytokine secretion was analyzed in BALF WT, Epac1-/-, Epac2-/- and PLCε-/- mice were exposed to air or CS for 4 days after which BALF was collected. KC (A), IL-6 (B) and IL-1β (C) levels were determined. Data are presented as mean ± SEM of 6-10 animals. $^{*}$ p<0.05, $^{**}$p<0.01, $^{***}$p<0.001 compared to basal control, $^{\#}$p<0.05, $^{\#\#}$p<0.01 compared to WT CS exposed.

**Role of Epac1-/-, Epac2-/- and PLCε-/- in mucus production**

Mucus production by goblet cells represents another remodeling parameter upon CS exposure (26; 27). Since the protein level of MUC5AC in all samples was below the detection level of the ELISA (not shown), we measured its expression by quantitative RT-PCR. In Epac1-/- and Epac2 -/- mice, expression of MUC5AC mRNA was significantly increased at basal level (Fig. 4A, Epac1-/- p<0.01, Epac2-/-

p<0.05), an effect not further enhanced upon CS exposure. The increase in MUC5AC mRNA was not observed in WT and PLCε-/- (Fig. 4A). In Epac1-/-, the higher expression of MUC5AC mRNA was accompanied by a higher mRNA expression of IL-13, known to be capable to increase in mucus production (27) . Next, we studied the numbers of goblet cells as main mucus producing cells in the epithelium (26; 27). We also analyzed the expression of an important factor of goblet cell differentiation, SPDEF (SAM pointed domain ETS factor) (28). Basal numbers of goblet cells were statistically different in PLCε-/- compared to WT, Epac1-/- and Epac2-/- mice (Fig. 4B; Supplemental Fig. 2B&D), Staining for SPDEF tended to be increased in Epac1-/- mice only (p=0.065; Fig. 4C and Supplemental Fig. 4D).

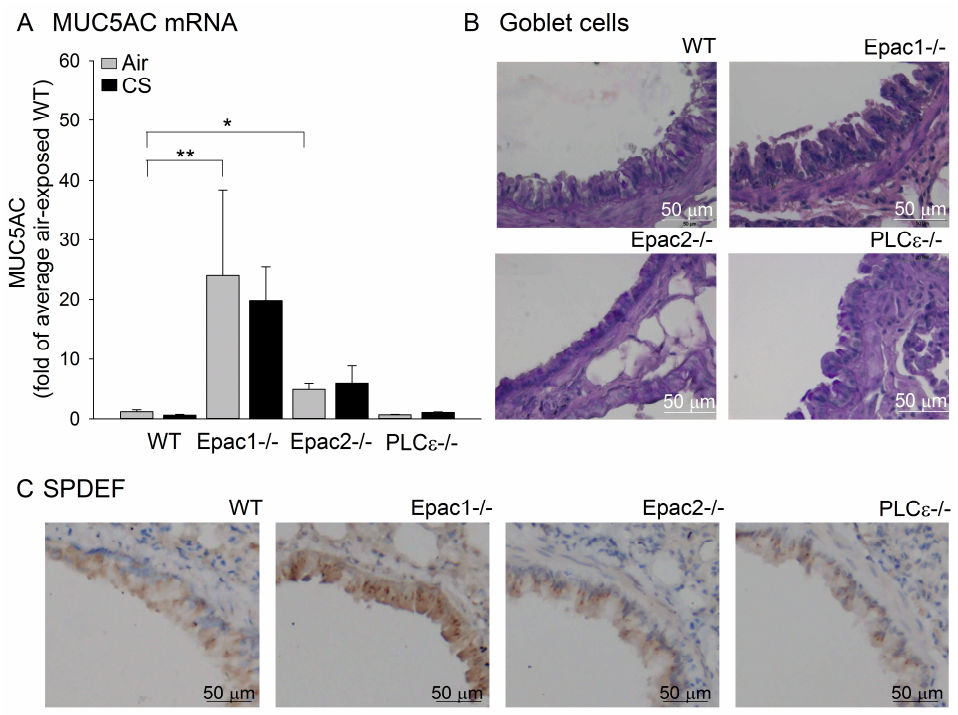



**Figure 4:** Analysis of MUC5AC mRNA expression, goblet cells and SPDEF positive cells. Regulation of MUC5AC mRNA, goblet cell number and SPDEF expression was analyzed in WT, Epac1-/-, Epac2-/- and PLC ε-/- mice. Expression of MUC5AC mRNA (A) was determined in lung homogenates of WT, Epac1-/-, Epac2-/- and PLCε-/- mice exposed to air or CS for 4 days. Representative pictures of PAS staining (B) and SPDEF

immunohistochemistry staining (C) are shown. In A, data are presented as mean ± SEM of 6-10 animals. * p<0.05, ** p<0.01, *** p<0.001 compared to basal control, # p<0.05 compared to WT exposed to CS.

**Role of Epac1-/-, Epac2-/- and PLCε-/- in matrix remodeling**

Finally, we studied mRNA expression of three remodeling parameters: the matrix proteins fibronectin, collagen I and the pro-fibrotic cytokine TGF-β1 (1; 29-31). Interestingly, basal levels fibronectin and TGF-β1 mRNA were increased in Epac1-/- compared to WT mice (Fig 5A&C, p<0.05). Fibronectin was further up-regulated by CS (Fig 5A, p< 0.01). Collagen mRNA expression in Epac1-/- mice was also higher after CS exposure compared to WT mice (Fig. 5B; p<0.01). No differences in fibronectin, collagen I and TGF-β1 were observed in the Epac2-/- and PLCε-/- strains compared to WT mice at basal levels (Fig. 5A-C).

Concerning protein expression, fibronectin and collagen I levels were similar for WT, Epac2-/- and PLCε-/- mice treated with either air or CS (Fig. 6A&B). Fibronectin and collagen I were significantly increased in Epac1-/- mice at basal level (Fig. 6B, p<0.01, p<0.001), effects not further enhanced upon CS exposure in Epac1-/- mice (Fig. 6A&B).

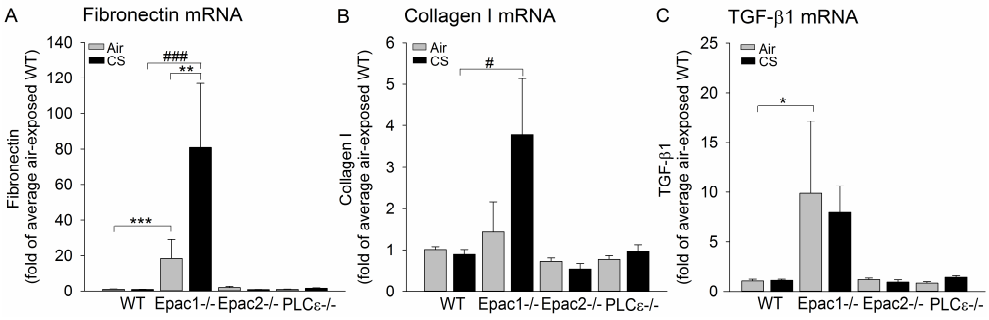



**Figure 5:** Determination of mRNA expression of remodeling parameters in total lung homogenates. Lung tissue of WT, Epac1-/-, Epac2-/- and PLCε-/- mice exposed to air or CS was homogenized and mRNA expression of fibronectin (A), collagen I (B) and TGF-β1 (C) was measured. Data are presented as mean ± SEM of 6-10 animals. ** p<0.01, *** p<0.001 compared to basal control, # p<0.05, ## p<0.01 compared to WT exposed to CS.

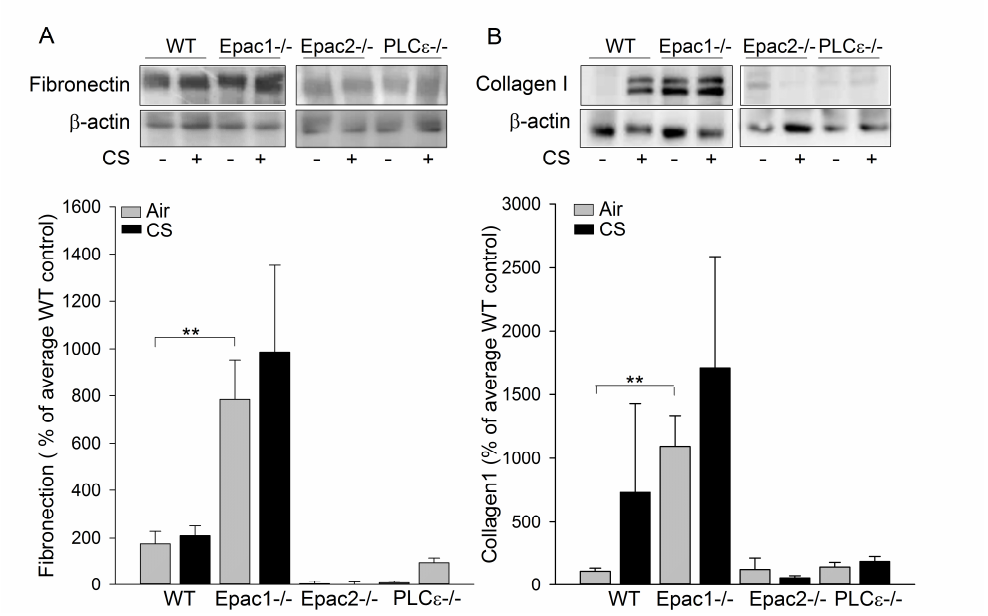



**Figure 6:** Protein expression of remodeling parameters was determined in total lung homogenates. Lung tissue of WT, Epac1-/-, Epac2-/- and PLCε-/- mice exposed to CS was homogenized and protein expression of fibronectin (A), and collagen I (B) was measured. Data are presented as mean ± SEM of 4-6 animals. $^{**}p<0.01$, $^{***}p<0.001$ compared to basal control.

**Discussion**

Here we report for the first time on a distinct role for Epac1 and Epac2 in remodeling and inflammatory processes *in vivo* using an acute model of 4 day exposure to CS. We report here that MUC5AC and matrix remodeling parameters, such as TGF-β1, collagen I and fibronectin, were increased in total lung homogenates of Epac1-/- mice at baseline. In particular, the increase in both fibronectin and collagen I protein point to a basal pro-fibrotic phenotype primarily in Epac1-/-. Regarding mucus production, we show that basal MUC5AC mRNA expression is regulated by both Epac1 and Epac2. In addition, our study shows that Epac2 (presumably both dependent and independent of PLCε) acts primarily pro-inflammatory in the acute mouse model of CS-induced lung inflammation (Fig. 7). Using Epac1-/-, Epac2-/- and PLCε-/- mice, we demonstrate that Epac2 and PLCε, contributes to CS-induced inflammatory responses, such as IL-6 secretion and increase in neutrophils numbers. Independently from PLCε, Epac2 regulates macrophage numbers and IL-1β secretion upon CS exposure. Epac1 exerts no profound effects on the mentioned inflammatory responses. In contrast, only Epac1 is capable of preventing the induction of TGF-β1, collagen I and fibronectin.

Exposure of WT mice to CS failed to induce MUC5AC, TGF-β1, fibronectin and collagen I pointing to the constraints of the short-term model being used in our current study. In line with this hypothesis, it has been reported that exposure of mice for 2 weeks to CS induced up-regulation of pulmonary vascular matrix metalloproteinases (32). In addition, pro-fibrotic remodeling processes were observed in a chronic model in mice exposed to LPS, a possible component of cigarette smoke (33). Importantly, however, studies in total lung homogenates of Epac1-/- mice on the level of matrix proteins pointed to an pro-fibrotic phenotype at baseline. Next to MUC5AC, epithelial wall thickness and SPDEF staining were altered in Epac1-/- mice suggesting that primarily Epac1-/- exhibit an interesting genotype at baseline. Overall our studies in an acute model of 4 day exposure to CS unravel distinct biological functions of Epac1 and Epac2 *in vivo*.

Our data show that C57BL/6J mice exposure to CS for 4 days evoked an inflammatory response primarily characterized by increase in macrophages and

neutrophils numbers as also observed in other studies (14; 34; 35). We demonstrate that Epac2 and PLCε are associated with an increase of neutrophils in the lung, whereas Epac2 alone is associated with an increase of macrophages. In addition, in our *in vivo* study we now show for the first time that Epac2 contributes to the increase of leukocytes into the BALF of mice exposed to CS. We also report here that the presence of neutrophils relies on PLCε, a known effector of Epac2 (9). This is in line with a study by Oka and colleagues, showing that PLCε increases ultraviolet-induced neutrophil numbers associated with skin inflammation in mice (11).

Our results also indicate that Epac2, but not Epac1, plays a role in the secretion of IL-1β upon CS exposure. In addition, we show that the levels of macrophages were lower in Epac2-/- mice exposed to CS. As IL-1β is the major cytokine secreted by macrophages (36), we hypothesize that Epac2 regulates the level of IL-1β in the BALF via increased numbers of macrophages. In agreement with our results on cytokine release, Tan and colleagues demonstrated that pharmacological activation of Epac in general induced an increased IL-1β production and similar effects were obtained upon activation of the $\beta_2$-adrenoceptor with salmeterol (37). The higher number of neutrophils in the BALF of CS-exposed mice, is most likely related to an increase of KC, a chemoattractant for neutrophils (38). Indeed, we observed a correlation between numbers of neutrophils and the levels of KC in WT and Epac2-/- mice, but not in Epac1 -/- and PLCε-/- mice. In these knockout mice other factors than KC seem to be involved in neutrophilia.
Next to the neutrophil chemoattractant KC and the macrophage-secreted IL-1β, we also show that IL-6 is increased in WT mice exposed to CS, an effect less pronounced in Epac2-/- mice. Thus, reduction of both neutrophils and macrophages was paralleled by a reduction in IL-1β and IL-6 levels in CS-exposed Epac2-/- mice. Besides Epac2, we show that PLCε is involved in CS-induced IL-6 secretion. Accordingly, Takenaka and colleagues demonstrated that PLCε elevates the expression of IL-6 mRNA in a skin inflammation model (39). Our data suggest that Epac2 and PLCε, induces an inflammatory response consisting of increased neutrophil numbers and IL-6 release.

Our previous work in human airway smooth muscle cells showed that Epac exerts an anti-inflammatory role in the CS-stimulated secretion of IL-8. We assigned the inhibition of IL-8 secretion to Epac1, due to a CS extract-induced reduction of Epac1 expression in these cells and a specific loss of Epac1 in total lung tissue of COPD patients (5). However, the regulatory function of Epac1 on inflammation is strictly cell-type dependent (4; 6; 40). Indeed, and most important, using Epac2-/- mice exposed to CS we identified this Epac isoform as the major regulator of airway inflammation *in vivo*.

Modulation of inflammatory responses by cAMP may involve PKA and Epac, thereby leading to the induction of pro- and/or anti-inflammatory effects (41; 42). As demonstrated in our model, CS did not profoundly alter the expression of the mRNA of components of the cAMP pathway in the lung, including Epac1 and Epac2 (mRNA and protein). The protein expression of Epac1 in Epac2-/- mice and that of Epac2 in Epac1-/- mice is not altered by CS, indicating the lack of compensatory mechanisms in case of absence of Epac1 or Epac2. Although most inflammatory responses are thought to be PKA-dependent, recent research points to a role in inflammation for Epac (reviewed in (43)) in line with our data.

Next to inflammation, CS can also induce airway fibrosis (44). Here, we report not only on distinct roles for Epac1 and Epac2 in inflammatory responses, but also in remodeling processes. Mice exposure for 4 days to CS might represent the most obvious limitation to induce changes in ECM protein expression and might also explain the variation being observed after exposure of WT mice to CS exposure. Importantly, however, the four mouse strains show differences at basal level concerning the expression of ECM protein expression. Indeed, Epac1-/- mice show higher levels of TGF-β1 (mRNA), collagen I (mRNA and protein) and fibronectin (mRNA and protein) at basal level. Based on our present findings reported here, we propose that Epac1-/- has a pro-fibrotic phenotype (Fig.7). This is in line with earlier reports showing that the cAMP effectors PKA and Epac inhibited the proliferation of fibroblasts and the production of the ECM proteins such as collagen I and III (45). In addition, TGF-β1 decreased the cellular level of Epac1 (7; 45). Conrotto and colleagues showed that Epac1 binds to the activated TGF-β1 type I receptor and subsequently decreases the phosphorylation of Smad2 and

Smad2-dependent transcription (46). These findings raise the possibility that Epac1 exerts its effect on collagen production via TGF-β1 (47). Also, as a further support, Yokoyama and colleagues showed that adenoviral overexpression of Epac1 inhibits TGF-β1 induced synthesis of collagen(7).

Mucus hypersecretion by mucus producing goblet cells is another factor involved in remodeling effects in the airways upon CS exposure (26; 27; 44). Most likely due to the short-term exposure to CS, we failed to observe induction of mucus secretion. However, the four mouse strains showed differences in basal characteristics concerning mucus secretion. Absence of Epac1 and Epac2 (less pronounced), but not PLCε, is associated with a constitutively higher expression of MUC5AC mRNA at basal level. The elevation of MUC5AC mRNA in Epac1-/- mice at basal level was accompanied by an increased secretion of IL-13, the latter known to promote mucus production (27). Goblet cells were increased in PLCε-/- mice, whereas primarily Epac1-/- mice tended to stain positive for the inducer of goblet cell differentiation SPDEF (26; 27).

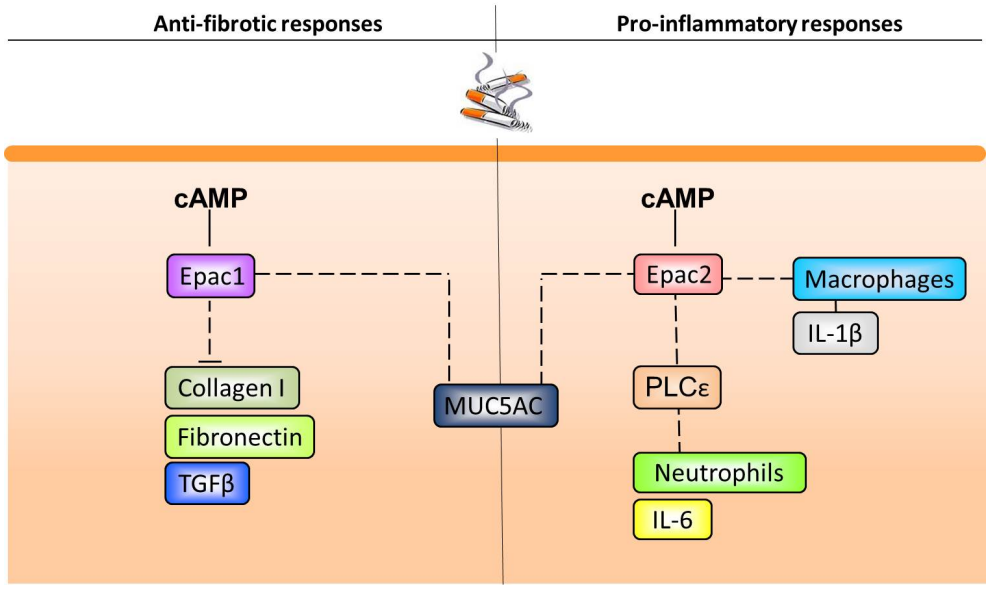



**Figure 7:** Proposed model for the regulation of inflammation and remodeling by Epac1, Epac2 and PLCε. Epac1 acts as an anti-fibrotic factor and inhibits the expression of fibronectin, collagen I, and TGF-β1. Epac1 and Epac2 inhibit the expression of MUC5AC. Epac2 regulates the number of CS-induced macrophages and IL-1β release. Presumably via PLCε, Epac2 regulates the number of CS-induced neutrophil numbers and IL-6 release. For details, see text.

The Epac effector PLCε is in this study specifically involved in inflammation processes, but not in remodeling processes. Accordingly, PLCε has been reported to promote via another effector of Epac, NF-κB, neuroinflammation (9; 48). Next to specific effectors of Epac, different compartmentalization mechanisms may be responsible for the differential effects of Epac1 and Epac2 as described. In this line, molecular complexes between the nuclear envelope-associated muscle AKAP (mAKAP), PDE4D3 and Epac1 (49), plasma membrane-associated AKAP5 and Epac2 (50), as well as mAKAP and PLCε (51) seem to induce a localized responses of Epac1 and Epac2 (49) resulting in a specific biological effect.

The pro-inflammatory role of cAMP-regulated Epac2 seems to be contradicting in the context of anti-inflammatory effects of cAMP-elevating drugs. However, it is uncertain whether cAMP-elevating agents decrease or increase airway inflammation most likely based on agent- and cell-type specific issue. The pro-inflammatory role of Epac2 may dampen anti-inflammatory effects by cAMP. Compartmentalization of cAMP, a process known to involve AKAP family members (52), may regulate specific activation of cAMP effectors to further fine tune cAMP signaling. Indeed, recently we showed that AKAP-based multiprotein complexes are present in bronchial epithelial cells (16). Currently, however, the specific effectors of Epac1 or Epac2 or the scaffold proteins involved in Epac1 or Epac2 signaling are unknown and should be a field of future research to unravel the distinct mechanisms used by Epac1 and Epac2.

Taken together, we show that *in vivo* Epac1 acts primarily anti-fibrotic while Epac2 and PLCε acts primarily pro-inflammatory. Our novel findings further emphasize that future research should aim to assign distinct biological functions to Epac1 and Epac2 in disease models, such as COPD. Design of subtype specific activators of Epac1 and Epac2 will most likely be of benefit to further unravel potentially distinct roles of Epac1 and Epac2 in *in vivo* settings.

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